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Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins

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ABSTRACT

Bacillus thuringiensis Vip3 proteins are synthesized and secreted during the vegetative growth phase. They are activated by gut proteases, recognize and bind to midgut receptors, form pores and lyse cells. We tested the susceptibility to Vip3Aa and Vip3Ca of Cry1A-, Cry2A-, Dipel- and Vip3-resistant insect colonies from different species to determine whether resistance to other insecticidal proteins confers cross-resistance to Vip3 proteins. As expected, the colonies resistant to Cry1A proteins, Dipel (*Helicoverpa armigera*, *Trichoplusia ni*, *Ostrinia furnacalis* and *Plodia interpunctella*) or Cry2Ab (*H. armigera* and *T. ni*) were not cross-resistant to Vip3 proteins. In contrast, *H. armigera* colonies resistant to Vip3Aa or Vip3Aa/Cry2Ab showed cross-resistance to the Vip3Ca protein. Moreover, the Vip3Ca protein was highly toxic to *O. furnacalis* (LC_{50} not significantly different from that of Cry1Ab), whereas the Vip3Aa protein only showed moderate growth inhibition at the highest concentration tested (100 µg/g of diet). These results extend the cross-resistance studies between Vip3 and Cry proteins, show for the first time cross-resistance between proteins within the Vip3 subfamily, and points to *O. furnacalis* as a target for the Vip3Ca protein.

1. Introduction

Vip3 insecticidal proteins are synthesized by *Bacillus thuringiensis* (Bt) during the vegetative growth phase and are active against lepidopteran insects (Chakroun et al., 2016a; Estruch et al., 1996). Vip3 proteins are classified into three protein subfamilies based on their amino acid sequence identity: Vip3A, Vip3B, and Vip3C (Crickmore et al., 2013). Most studies on the insecticidal activity of Vip3 proteins have been performed on the Vip3A protein subfamily, in particular, with the Vip3Aa protein. Vip3Ca was discovered more recently and show some toxic effect against some lepidopteran species (Palma et al., 2012; Gomis-Cebolla et al., 2017). In contrast to Vip3Ca, Vip3A proteins have a broad insecticidal spectrum against lepidopteran pests (Chakroun et al., 2016a). The fact that the insecticidal spectrum and the mode of action of the Vip3 proteins differ from that of the Cry1 and Cry2 proteins, makes Vip3 proteins good candidates to be used in combinations with Cry proteins in Insect Resistance Management (IRM) programs.

The mode of action of Vip3 proteins (Vip3A and Vip3C) shares some similarities to that of the Cry proteins in that they are synthesized in the form of protoxins, which are further processed by midgut proteases rendering the active toxin (Estruch et al., 1996; Yu et al., 1997; Lee et al., 2003; Chakroun et al., 2012; Caccia et al., 2014; Gomis-Cebolla et al., 2017). The activated toxins bind to specific receptors in the midgut membrane leading to the disruption of the midgut epithelial cells and eventual death of the larva. The Vip3 receptors are not shared by Cry proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun et al., 2014; Gomis-Cebolla et al., 2017). However, it has been recently shown that the Vip3Aa and Vip3Ca proteins compete for shared binding sites (Gomis-Cebolla et al.,

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In agriculture worldwide, repeated applications of *Bt* sprays and widespread adoption of *Bt*-crops (transgenic crops protected from insects by the expression of *cry* or/and *vip3* genes) have led to resistance (Ferré and Van Rie, 2002; Ferré et al., 2008, Tabashnik, 2015; Tabashnik et al., 2009). Therefore, in this arms race against insects, it is necessary to keep exploring the potential of new insecticidal proteins for pest control and, at the same time, to test for their compatibility in combinations with other proteins in terms of cross-resistance. Although cross-resistance studies have been performed with Cry1- and Cry2-resistant colonies against Vip3Aa (Jackson et al., 2007; Fang et al., 2007; Anilkumar et al., 2008; Vélez et al., 2013; Huang et al., 2014; Qian et al., 2015; Welch et al., 2015; Horikoshi et al., 2016), and with Vip3Aa-resistant colonies against Cry1A proteins (Mahon et al., 2012; Chackroun et al., 2016b; Pickett et al., 2017), cross-resistance to Vip3Ca has never been tested.

In this study, we tested the susceptibility of Cry1-, Cry2- and Dipel-resistant colonies from four insect species (*Trichoplusia ni*, *Plodia interpunctella*, *Helicoverpa armigera* and *Ostrinia furnacalis)* to the Vip3Aa and Vip3Ca proteins and compared the results to the non-selected controls. In addition, we tested two Vip3Aa-resistant colonies from *H. armigera* for cross-resistance to Vip3Ca.

2. Materials and methods

2.1. Insect colonies

2.1.1. Insect rearing of T. ni strains

Three *T. ni* strains were used to examine their response to Vip3Aa and Vip3Ca. The *T. ni* Cornell laboratory strain (Wang et al., 2007) was used as the susceptible control strain. The two resistant strains were a Cry1Ac-resistant strain, GLEN-Cry1Ac-BCS (Wang et al., 2007), and a Cry2Ab-resistant strain, GLEN-Cry2Ab-BCS (Song et al., 2015). Both of the resistant *T. ni* strains were near-isogenic to the susceptible Cornell strain and the resistance is fixed (i.e., they were homozygous for the resistance genes). The *T. ni* colonies were maintained on artificial diet without exposure to Bt toxins (Bell et al., 1981).

2.1.2. Insect rearing of H. armigera strains

Five *H. armigera* strains were used to determine their response to Vip3Ca. The *H. armigera* susceptible colony, GR, was used as a control (Mahon et al., 2007). The *H. armigera* homozygous resistant colonies ISOC8, (Cry1Ac), Sp15 (Cry2Ab resistant) and Sp85 (Vip3A resistant) were established from lab selection (ISOC8) and positive F_2 tests in 2002 (Sp15) and 2010, (Sp85) respectively. The *H. armigera* Cry2Ab/Vip3A resistant strain was established by reciprocal crosses placing male pupae from one strain with female pupae from the other in cages (Walsh et al., 2014). All of the resistant strains were repeatedly outcrossed to a susceptible colony and reselected with the appropriate toxin(s). The Cry2Ab resistant line Sp15 carries an ABCA2 transporter mutation that confers the phenotype (Tay et al., 2015), but the mechanism of resistance is unknown for ISOC8 (Cry1Ac) and Sp85 (Vip3A). The rearing methods used to maintain *H. armigera* were modified from those described by Teakle and Jensen (1985).

2.1.3. Insect rearing of P. interpunctella strains

Two *P. interpunctella* strains were used to characterize their response to Vip3Aa. The *P. interpunctella* susceptible colony, EP, was obtained from a grain storage bin and has been maintained in the laboratory on cracked wheat diet (Oppert et al., 2010). The resistant colony EP-Dpl500 was selected from the parental EP, with 500 mg Dipel (Bt subspecies *kurstaki*, strain HD-1) per kg diet, gradually increasing the dose to 10,000 mg/kg, the maintenance dose for this resistant colony.

2.1.4. Insect rearing of O. furnacalis strains

Two strains of *O. furnacalis*, a Bt susceptible strain and a Cry1Ab-resistant strain were established in the laboratory. The Bt susceptible strain was collected from the field and had been reared using standard rearing techniques without exposure to any insecticide before bioassays were conducted (Song et al., 1999). The Cry1Ab-resistant strain was selected from the Bt susceptible strain by exposure to trypsin-activated Cry1Ab. The Cry1Ab-resistant strain was initially exposed throughout larval development to Cry1Ab in the artificial diet (2.5 ng of toxin/g diet). The toxin concentration was increased in succeeding generations to target 40–70% mortality in the exposed insects. After 51 generations, larvae were reared on diet containing 400 ng of toxin/g diet. The Cry1Ab-selected colony had developed > 100-fold resistance to Cry1Ab after 35 generations (Xu et al., 2010).

2.2. Source and expression of Vip3 proteins for cross-resistance assays

Vip3Aa (NCBI accession No AAW65132) was overexpressed in recombinant *Escherichia coli* BL21 carrying the *vip3Aa16* gene (Abdelkefi-Mersati et al., 2009). The Vip3Ca protein (NCBI accession No AEE98106) was prepared from recombinant *E. coli* WK6 carrying the *vip3Ca2* gene (Palma et al., 2012).

The Vip3Aa protein was expressed following the conditions described by Chakroun et al., 2012. For the Vip3Ca protein, a single colony was inoculated in 7 ml of LB medium containing 100 µg/ml ampicillin and grown overnight at 37 °C and 180 rpm. A 1/100 dilution of the culture in 700 ml LB medium containing 100 µg/ml ampicillin was further incubated at 37 °C and 180 rpm. The culture was induced with 1 mM IPTG at an OD of 0.7 and it was grown overnight at 37 °C and 200 rpm. Cells were collected at 6000 g for 15 min at 4 °C. The pellet was weighed and suspended in 3 ml lysis buffer (PBS, pH 8.0, containing 3 mg/ml lysozyme, 10 µg/ml DNase, and 100 µM PMSF) per gram of pellet. The sample was incubated at 37 °C for 30 min and then sonicated on ice applying two 1 min pulses at 70 W at a constant duty cycle, separated by a 10-s cooling period on ice. Then, the insoluble material was separated by centrifugation at 16,000 g for 15 min at 4 °C and the soluble cellular fraction sequentially filtered through sterile 0.45 µm and 0.22 µm cellulose acetate filters.

2.3. Purification of Vip3 proteins for cross-resistance assays

Vip3 proteins used for dose-response assays for the *T. ni*, *H. armigera* and *O. furnacalis* colonies were purified by isoelectric point precipitation (IPP) (Chackroun et al., 2012; Gomis-Cebolla et al., 2017). The pH of the lysate was lowered with acetic acid to pH 5.5 for Vip3Aa and pH 5.95 for Vip3Ca. The pellets were recovered by centrifugation at 16,000 g for 10 min and then dissolved in 20 mM Tris, 150 mM NaCl, pH 9, and dialyzed against the same buffer overnight. The Vip3Aa protein used for dose-response assays for the *P. interpunctella* colonies was purified by immobilized metal ion absorption chromatography (IMAC) on a Hi-Trap chelating HP column (GE Healthcare) charged with Ni^{2+} (Fig. 1A) (Chackroun et al., 2012). The purified proteins were frozen at −80 °C and then lyophilized. The concentration of the Vip3 proteins purified by IPP was estimated by densitometry after SDS-PAGE separation. The concentration of the Vip3Aa purified by Hi-Trap chelating HP column was measured by the method of Bradford (Bradford et al., 1976). In both methods, bovine serum albumin (BSA) was used as standard. The purity of the Vip3 proteins was analyzed by SDS-PAGE (Fig. 1).

2.4. Insect toxicity assays

2.4.1. Dose-response assays for the susceptible and resistant T. ni strains

Examination of *T. ni* strains for their susceptibilities to Vip3Aa and Vip3Ca were conducted using the surface contamination method (Kain et al., 2004). Briefly, an aliquot of 200 µl of Vip3Aa or Vip3Ca solution was spread on the surface of diet in 30-ml cups (surface area is approximately 7 cm^2) and 10 neonate larvae were placed in each cup that

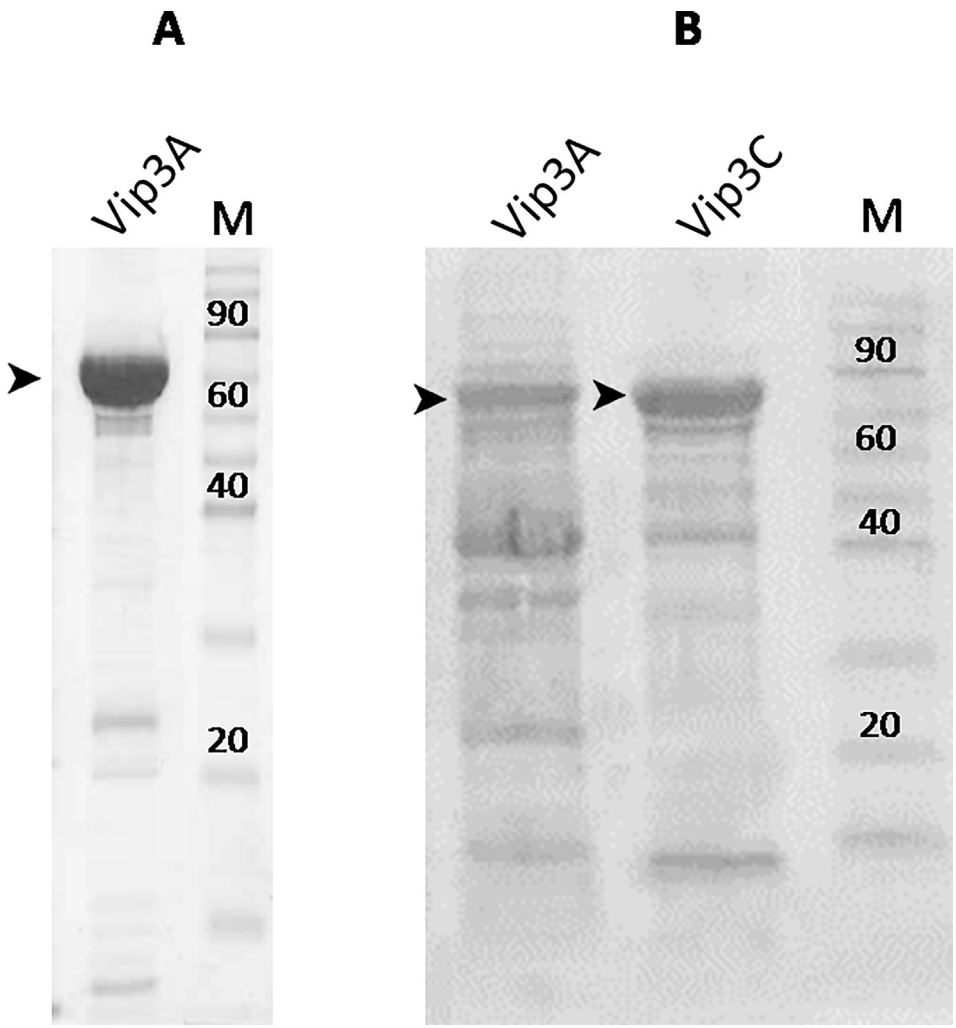

Fig. 1. SDS-PAGE of Vip3Aa and Vip3Ca protoxins after partial purification from *E. coli* extracts. (a) Vip3Aa purified by Hi-Trap chelating HP column charged with Ni^{+2}. (b) Vip3Aa and Vip3Ca purified by isoelectric point precipitation. M: Molecular Weight Markers ("PINK PlusPrestained Protein Ladder", from Genedirex). The arrowhead indicates the band corresponding to the Vip3 protein.

was covered with a lid and placed at 27 °C. Eight concentrations of Vip3Aa or Vip3Ca in 2-fold serial dilutions from 800 to 6.25 μg/ml and a non-toxin control were used for each bioassay. Five replicate cups of larvae (50 larvae in total for each dose) were included for each concentration of toxin. Larval mortality and growth inhibition (larval developmental stage remaining in the first instar) were recorded after 4 days of rearing on diet. The bioassay data were subjected to Probit analysis using the software POLO, LeOra Software to obtain the LC_{50} and IC_{50} of the Vip proteins (Russell et al., 1977).

2.4.2. Dose-response assays for the susceptible and resistant H. armigera strains

The susceptibility of *H. armigera* strains to Vip3Aa and Vip3Ca was conducted using the surface contamination method as follows. Approximately 300 μl of standard diet was added to straight sided 96 well trays providing 0.567 cm^2 of surface area. Once the diet had cooled, 20 μl of a solution containing an appropriate concentration of toxin was added and allowed to air dry. One neonate was placed in each well before it was sealed with a perforated heat-sensitive lid. Trays were incubated at 25 °C, 60% RH, and 14:10 h light: dark for 8 days. Six concentrations of Vip3Ca in 2-fold serial dilutions from 20 to 0.625 μg/cm^2 and a non-toxin control were used for each bioassay. A minimum of three replicate bioassays of larvae (16 larvae in total for each dose) were included for each concentration of toxin. The numbers of dead and alive larvae were counted and the data were subjected to Probit analysis using the software POLO, LeOra Software to obtain the LC_{50} of the Vip proteins (Russell et al., 1977). Moreover, the instars of surviving larvae were recorded to obtain the larval development index (LDI) of surviving larvae.

$$LDI = \frac{[(nL1 \times 1) + (nL2 \times 2) + (nL3 \times 3) + (nL4 \times 4) + (nL5 \times 5)]}{N}$$

where nL1, nL2, nL3, nL4 and nL5 are the numbers of alive larvae in the respective larvae development stage and N refer the total number of alive larvae. We consider as statistically significant all those LDI values where the 95% confidence intervals of the mean did not overlap with another LDI value for the susceptible and resistant population (Cry1Ac-, Cry2Ab-, Vip3A- and Vip3/Cry2Ab-resistant) of *H. armigera*. Confirmation of resistance in the Cry1-, Cry2-, Vip3-, and Vip3/Cry2Ab-resistant insects at the time of Vip3 bioassays was obtained from single dose assays at a discriminant dose (Table S1).

2.4.3. Dose-response assays for the susceptible and resistant P. interpunctella strains

The *P. interpunctella* strains used in the current study are EP (Dipel-susceptible) from which EP-Dpl500 was selected with Dipel, and the parent strain for the Dipel-resistant strain which was selected and maintained on 10,000 mg/kg Dipel (the resistant strain used in this study). The Dpl500 strain was moderately resistant to Cry1F (18-fold) and Cry1Ab (over 100-fold), and very resistant to Cry1Ac (> 3000-

fold) and Dipel (with no mortality observed at the highest dose) (Table S2). The bioassays of *P. interpunctella* strains with Vip3 toxins were conducted using 4 mm round diet disks punched out from a flattened cereal mixture (Herrero et al., 2001). Briefly, 5 µl of 11 different doses of Vip3Aa (from 0.1 to 100 µg/4 mm diet disk for Dipel-susceptible insects, and from 2 to 100 µg/4 mm diet disk for Dipel-resistant insects) and Vip3Ca (from 2 to 100 µg/4 mm diet disk for Dipel-susceptible insects, and from 2 to 100 µg/4 mm diet disk for Dipel-resistant insects) were added to diet disks using a micropipettor, with controls of 5 µl water only for each replicate. Treated diet disks were placed in 16-well black assay trays (Frontier Agricultural Sciences, Newark, DE, USA), and eggs were added to each well (n = 16 individuals per dose, three independent biological replicates for each toxin and *P. interpunctella* strain). Trays were covered with perforated adhesive plastic sheets and incubated at 28 °C and 75% relative humidity (R.H.) in darkness. Mortality was recorded at approximately 21 d. Data was analyzed by Probit analysis using the software POLO, LeOra Software (Russell et al., 1977) to obtain the LC_{50} of the Vip proteins, reported in mg of toxin per 4 mm (15 mg) diet disk.

2.4.4. Dose-response assays for the susceptible and resistant O. furnacalis strains

The susceptibility of *O. furnacalis* neonates to Vip3 proteins (Vip3A and Vip3Ca) was determined in dose-response assays in agar-free semi-artificial diet (He et al., 2005). Briefly, a single neonate was randomly transferred into each well of 48-well tray and then covered with a piece of paper and the lid. Trays were held in a growth chamber for seven days at 27 °C, 80% RH and a 16:8 h photoperiod. Number of dead larvae and the weight of larvae surviving per tray were recorded after seven days of exposure. If a larva had not developed beyond the first instar and weighed ± 0.1 mg, it would be counted as dead for calculating practical mortality. Average larval weight of survivors would be used to determine the larval growth inhibition rate as a function of toxin concentration. Bioassays were repeated on two dates with total of 96 larvae per concentration and included 6–10 concentrations of purified toxin. Dilutions of Vip3 toxins were prepared in 20 mM Tris 150 mM NaCl, pH 9. The same buffer was used as a negative control. Bioassay data were subjected to Probit analysis using the software POLO, LeOra Software 1977 to obtain the LC_{50} of the Vip proteins (Russell et al., 1977). Confirmation of resistance in the Cry1Ab-resistant insects at the time of Vip3 bioassays was obtained from dose-response bioassays with Cry1Ab (Table S3).

3. Results and discussion

3.1. Cry1A and Dipel-resistant colonies

We first tested the vulnerability of susceptible and Cry1A and Dipel-resistant colonies (*P. interpunctella, T. ni*, *H. armigera* and *O. furnacalis*) against Vip3 proteins (Table 1). Previously it was demonstrated in *T. ni* that Cry1Ac-resistant strains were not cross-resistant to Vip3A proteins (Fang et al., 2007), but Vip3Ca had never been tested. Our results show that Vip3Ca is 5–10-fold less active than Vip3Aa to this insect species and that Cry1Ac-resistance does not confer cross-resistance to either of the two Vip3 proteins. However, growth inhibition assays showed that Cry1Ac-resistant insects were slightly less affected by both Vip3Aa and Vip3Ca (IC_{50} around 2-fold higher) compared to the susceptible insects (Table 2).

P. interpunctella had never been tested for cross-resistance to Vip3 proteins. Although the LC_{50} for Vip3Aa in the Dipel-resistant strain was approximately 5-fold higher than that of the Dipel-susceptible strain, the fiduciary limits were overlapping. Conversely, the Dipel-susceptible strain was approximately 5-fold less susceptible to Vip3Ca than the Dipel-resistant strain, but again with overlapping fiduciary limits. Therefore, cross-resistance to Vip3 proteins was not observed in the two *P. interpunctella* strains. In comparing responses of the two Vip3 toxins to Cry toxins, Dipel-susceptible insects were less susceptible to Vip3 than Cry toxins, whereas Dipel-resistant insects were more susceptible to Vip3 toxins than Dipel, Cry1F, and Cry1Ac (Table S2).

Lack of cross-resistance to Vip3Aa in Cry1Ac-resistant *H. armigera* colony has been previously shown (Mahon et al., 2012; Chackroun et al., 2016b) and, therefore, only Vip3Ca was used in our study. The LC_{50} values for the Cry1Ac-resistant versus susceptible colonies were statistically different, indicating that resistant insects were 3.3-fold more susceptible to Vip3Ca. This difference could reflect a cost in the Cry1Ac-resistant insects which makes them less fit to withstand the same doses of Vip3Ca. As with *P. interpuntella,* fitness costs in Cry1Ac-resistant *H. armigera* may induce increased susceptibility to other toxins, such as Vip3Ca. An assay with sublethal doses of Vip3Ca was performed to test whether the slightly higher mortality produced by this protein on Cry1Ac-resistant larvae was reflected in the development time. As is indicated in Table 3, no significant differences in the larval development index (LDI) were detected between susceptible and Cry1Ac-resistant population.

Regarding *O. furnacalis*, this insect species has never been tested before against Vip3 proteins. The results of *O. furnacalis* (both susceptible and Cry1Ab-resistant strains) with Vip3Aa show that this species is not susceptible to this protein (Table 1). Only at 100 µg/g diet about 50% growth inhibition was observed in the two strains. In contrast, Vip3Ca was highly active to *O. furnacalis* larvae from both strains. The LC_{50} value of Vip3Ca for the susceptible strain was not significantly different from that of Cry1Ab (LC_{50} = 0.23 µg/g, FL = 0.17–0.30) (Table S3). However, because of the different slope values of their respective regression lines (Tables 1 and S2), Vip3Ca has an LC_{90} value (LC_{90} = 0.98 µg/g, FL = 0.81–1.31) lower than that of Cry1Ab (LC_{90} = 2.94 µg/g, FL = 1.94–2.53), suggesting that the former is more effective to control this pest than the latter. The small difference (LC_{50} 3.12-fold and LC_{90} 3 fold) between Vip3Ca LC values of the susceptible and Cry1Ab-resistant insects is statistically different, suggesting that Cry1Ab-resistance in this strain confers minimum cross-resistance to Vip3Ca.

3.2. Cry2Ab-resistant colonies

Prior to this study, cross-resistance to Vip3 proteins was not found in two Cry2A-resistant colonies from *Heliothis virescens* (Jackson et al., 2007). In the present study, Cry2Ab-resistant colonies of *T. ni* and *H. armigera* were tested against Vip3 proteins, and the results were compared to their susceptible controls (Table 1). Cry2Ab-resistant *T. ni* was not cross-resistant to either of the two Vip3 proteins as measured by mortality (Table 1) or growth inhibition (Table 2).

Cry2Ab-resistant *H. armigera* had never been tested for cross-resistance to Vip3 proteins, though lack of cross resistance or genetic linkage with Cry2Ab-resistance loci had been reported for Vip3Aa-resistant colonies (Mahon et al., 2007; Mahon et al., 2012; Chackroun et al., 2016b). Similarly to Cry1Ac-resistant *H. armigera* colony, the Cry2Ab-resistant insects were slightly more vulnerable (2.3-fold) than susceptible ones (Table 1). As with Cry1Ac-resistant insects, sublethal doses of Vip3Ca did not drive differences in larvae development (Table 3).

3.3. Vip3Aa-resistant colonies

Cross-resistance within the Vip3 subfamily of proteins has not yet been established. We tested the susceptibility of two Vip3-resistant *H. armigera* colonies (one resistant to Vip3Aa alone and the other resistant toVip3Aa and Cry2Ab) against the Vip3Ca protein (Table 1). The highest Vip3Ca doses tested (20 µg/cm^2 for the Vip3Aa-resistant insects and 10 µg/cm^2 for the Vip3Aa/Cry2Ab-resistant insects) only caused a mortality of 4.7% and 6.2%, respectively. Compared to the mortality observed in the susceptible control insects and the Cry1- and Cry2-resistant insects, these results clearly indicate that resistance to Vip3Aa

Table 1
Evaluation of the susceptibility to Vip3 proteins of susceptible and Cry1-, Cry2-, Dipel- and Vip3-resistant insect colonies from different insect species.

Insect species	Protein tested	Replicates	Colony	Slope ± SE[1]	LC_{50} (FL_{95})[2]	Resistance[3] ratio
T. ni					µg/cm^2	
	Vip3Aa	R1[4]	Susceptible	4.0 ± 0.4	0.95 (0.84–1.09) a	–
			Cry1Ac-resistant	5.0 ± 0.6	1.41 (1.07–1.91) a	1.48
			Cry2Ab-resistant	2.7 ± 0.3	1.10 (0.51–1.77) a	1.15
		R2[4]	Susceptible	4.3 ± 0.5	1.05 (0.65–1.80)[5] a	–
			Cry1Ac-resistant	4.2 ± 0.4	2.16 (1.30–3.83)[5] a	2.05
			Cry2Ab-resistant	3.4 ± 0.4	1.63 (1.36–1.90) a	1.55
	Vip3Ca	R1[4,§]	Susceptible	2.5 ± 0.2	8.53 (5.42–15.09) [b]	–
			Cry1Ac-resistant	2.5 ± 0.3	10.02 (6.07–18.3) b	1.17
			Cry2Ab-resistant	1.6 ± 0.2	16.12 (8.33–82.09) b	1.88
		R2[4,§]	Susceptible	3.3 ± 0.4	3.02 (2.53–3.54) c	–
			Cry1Ac-resistant	2.9 ± 0.3	4.95 (2.82–8.33) c	1.63
			Cry2Ab-resistant	3.0 ± 0.3	4.05 (3.40–4.78) c	1.33
P. interpunctella					µg/15 mg diet disk	
	Vip3Aa		Susceptible	0.43 ± 0.09	15.9 (2.25 – 151) d	–
			Dipel-resistant	0.65 ± 0.16	78.7 (27.2–792) d	4.95
	Vip3Ca		Susceptible	0.58 ± 0.20	67.5 (5.87–394) e	–
			Dipel-resistant	0.42 ± 0.12	13.7 (1.04–76.0) e	0.20
H. armigera					µg/cm^2	
	Vip3Ca		Susceptible	2.1 ± 0.20	5.34 (4.34–6.65) f	–
			Cry1Ac-resistant	2.1 ± 0.30	1.60 (1.11–2.11) g	0.30
			Cry2Ab-resistant	1.9 ± 0.27	2.36 (1.70–3.14) g	0.44
			Vip3Aa-resistant	–	NA[6]	0
			Vip3Aa/Cry2Ab-resistant	–	NA[6]	0
O. furnacalis					µg/g	
	Vip3Aa		Susceptible	–	UD[7]	–
			Cry1Ab-resistant	–	UD[7]	0
	Vip3Ca		Susceptible	2.52 ± 0.37	0.31 (0.22–0.38)h	–
			Cry1Ab-resistant	1.67 ± 0.15	0.97 (0.74–1.22)i	3.12

[1] SE: Standard error of the slope.
[2] LC_{50} values followed by the same letter are not significantly different from their corresponding susceptible strain based on the overlap of fiducial limits (FL).
[3] Resistance Ratio was calculated dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain.
[4] The dose-response assays, R1 and R2, are biological replications. Within each biological replication, three *T. ni* strains were assayed using the same Vip solutions at the same time.
[5] FL_{90}, instead of FL_{95}, was calculated and presented, as the heterogeneity of the data was above the default threshold ($g > 0.5$) by POLO for adequate FL_{95} calculation.
[6] NA: Non active. The highest dose tested (20 µg/cm^2 for the Vip3-resistant and 10 µg/cm^2 for theVip3Aa/Cry2Ab-resistant) caused a mortality of 4.7% and 6.2%, respectively.
[7] UD: Unable to determine. The highest dose tested (100 µg/g for the susceptible and resistant *O. furnacalis* strains) there was not significant mortality observed. However, it showed about 50% growth inhibition.
[§] The differences observed in the Vip3 proteins were considered as variations between replicates.

Table 2
Evaluation of the growth inhibition to Vip3Aa and Vip3Ca of susceptible and Cry1-, Cry2-resistant *T. ni* insect colonies.

Insect species	Protein tested	Replication	Colony	Growth inhibition		Resistance ratio[4]
				Slope ± SE[1]	IC_{50} (FL_{95})[2]	
T. ni					µg/cm^2	
	Vip3Aa	R1[3]	Susceptible	5.5 ± 0.7	0.33 (0.30–0.37) a	–
			Cry1Ac-resistant	4.3 ± 0.5	0.64 (0.44 0.86) b	1.93
			Cry2Ab-resistant	4.0 ± 0.7	0.24 (0.18–0.29) a	0.72
		R2[3]	Susceptible	4.0 ± 0.5	0.30 (0.26–0.35) a	–
			Cry1Ac-resistant	3.6 ± 0.4	0.64 (0.55–0.73) b	2.10
			Cry2Ab-resistant	5.0 ± 0.9	0.27 (0.22–0.31) a	0.88
	Vip3Ca	R1[3]	Susceptible	3.4 ± 0.3	1.17 (1.00–1.35) c	–
			Cry1Ac-resistant	4.3 ± 0.5	2.20 (1.34–3.42) c	1.88
			Cry2Ab-resistant	4.2 ± 0.5	1.38 (1.21–1.57) c	1.18
		R2[3]	Susceptible	4.2 ± 0.7	0.41 (0.33–0.47) d	–
			Cry1Ac-resistant	3.8 ± 0.6	0.93 (0.75–1.09) c	2.28
			Cry2Ab-resistant	4.8 ± 0.7	0.54 (0.45–0.62) d	1.33

[1] SE: Standard error of the slope.
[2] IC values followed by the same letter are not significantly different based on the overlap of FL.
[3] The dose-response assays, R1 and R2, are biological replications. Within each biological replication, three *T. ni* strains were assayed using the same Vip solutions at the same time.
[4] Resistance Ratio was calculated dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain.

Table 3
Evaluation of the larval development index to Vip3Ca of susceptible and Cry1-, Cry2-, Vip3-, and Vip3/Cry2Ab-resistant *H. armigera* insect colonies.

Dose (μg/cm^2)	LDI[1] ± CI[2]				
	Susceptible	Cry1Ac-resistant	Cry2Ab-resistant	Vip3A-resistant	Vip3A/Cry2Ab-resistant
20	1.06 ± 1.25	0.62 ± 2.00	0.00 ± 0.00	3.58 ± 2.86	–
10	1.00 ± 1.24	0.83 ± 3.59	1.09 ± 4.69	3.73 ± ND*	3.56 ± 5.53
5	2.00 ± 1.17	1.72 ± 3.96	2.10 ± 2.24	3.9 ± ND*	3.60 ± 5.15
2.50	2.70 ± 0.61	2.27 ± 1.87	2.66 ± 2.58	3.9 ± ND*	3.60 ± 5.10
1.25	2.54 ± 0.56	2.83 ± 1.84	2.87 ± 2.93	3.9 ± ND*	3.62 ± 4.77
0.60	3.05 ± 0.56	3.05 ± 1.80	3.12 ± 1.83	3.9 ± ND*	3.80 ± 2.80
Controls (μg/cm^2)					
0	3.60 ± 0.63	3.50 ± 1.29	3.78 ± 1.28	3.20 ± 2.48	3.65 ± 4.44
Cry1Ac[3]	0.16 ± 0.43	2.97 ± 0.54	0.00 ± 0.00	0.0 ± 0.0	0.00 ± 0.00
Cry2Ab[3]	0.17 ± 0.43	1.00 ± 0.00	3.80 ± 0.94	0.0 ± 0.0	3.60 ± 5.10
Vip3A[3]	0.67 ± 0.54	0.66 ± 2.87	1.00 ± 0.00	2.90 ± 1.20	3.40 ± 6.35

[1] Larval development index calculated as: LDI = [1 × (Number of L1 larvae) + 2 × (Number of L2 larvae) + 3 × (Number of L3 larvae) + 4 × (Number of L4 larvae) + 5 × (Number of L5 larvae)]/Total of alive larvae. Differences between LDI values from the susceptible and resistant populations were considered statistically significant if the 95% confidence intervals (CI) of the mean did not overlap.

[2] CI: Confidence interval of the mean.

[3] Discriminant dose for Cry1Ac (0.25 μg/cm^2), Cry2Ab (0.25 μg/cm^2), and Vip3Aa (20 μg/cm^2), respectively.

* ND: Not possible to calculate the 95% CI of the Vip3A-resistant *H. armigera* colony because the assay was done with one replicate.

conferred cross-resistance to Vip3Ca. This conclusion is supported by the results in Table 3, where Vip3Ca did not cause any delay in the development of the two Vip3Aa-resistant colonies.

The results obtained regarding cross-resistance are in agreement with the differences in the mode of action of Cry and Vip3 proteins. Several studies demonstrated that Vip3Aa does not share binding sites with Cry1 or Cry2 proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun et al., 2014) and recent work showed that Vip3Aa and Vip3Ca share binding sites in *Mamestra brassicae* (Gomis-Cebolla et al., 2017). Some of the Cry-resistant insect colonies evaluated in this study had previously been examined for alteration in Cry protein binding and reduced binding was found for the Cry proteins used as the selective agent. For example, Cry1Ac toxins did not bind to gut membrane proteins in the Cry1Ac-resistant *T. ni* colony (Wang et al.; 2007), gut proteins from a Dipel-resistant *P. interpunctella* colony had drastically reduced binding of Cry1Ab (Herrero et al., 2001), and gut proteins from the Cry2Ab-resistant *H. armigera* colony had a greatly reduced binding of Cry2Ab (Caccia et al., 2010). In the case of *O. furnacalis*, no binding assays have been reported with resistant strains. Although binding is not the sole mechanism of resistance to Bt toxins, it is the one that confers more specific and higher levels of resistance (Ferré and Van Rie, 2002; Ferré et al., 2008).

Despite the fact that Vip3Aa and Vip3Ca have been shown to share binding sites in *M. brassicae* (Gomis-Cebolla et al., 2017), our cross-resistance result is not straightforward to explain, Vip3Aa binding apparently was not affected in the Vip3Aa-resistant *H. armigera* colony (Chackroun et al., 2016b). We can think of several scenarios to explain this apparent paradox. First, the binding of Vip3Aa to a "functional" receptor (by this we mean a membrane molecule that triggers the subsequent steps to kill the cell) can be masked by binding to other molecules in the membrane. Examples have been reported for Cry proteins, such as the case of Cry1Ac in *H. virescens*, which binds to three binding sites while only binding site A is responsible for toxicity and, thus, reduced Cry1Ac binding is not detected in resistant insects (Lee et al., 1995; Jakka et al., 2015). Second, assuming that no brush border epitope has been altered in the Vip3Aa-resistant insects, the mechanism of resistance may lay in successive steps, such as membrane insertion, pore formation, or any other post-binding event such as a signal transduction leading to cell death. If this was the case, our results would indicate that Vip3Aa and Vip3Ca, besides sharing a binding epitope in the brush border membrane, share a post-binding step which would be impaired in resistant insects. Whichever the case, our cross-resistance results indicate these two Vip3 proteins share common steps in the mode of action.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jip.2018.05.004.

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